

Application of ELISA for assessment of antiserum immunoreactivity in endocrine immunocytochemical studies

AC NIEUWENHUIJZEN KRUSEMAN

From the Departments of Endocrinology, St Bartholomew's Hospital, London EC1, and University Hospital, Leiden, The Netherlands

SUMMARY An indirect method of an enzyme-linked immunosorbent assay (ELISA) is described to assess the reactivity of antisera used for the identification of peptide hormone producing cells by immunocytochemistry. Compared with radioimmunoassay and immunodiffusion, the ELISA method has the advantages of simplicity and sensitivity and represents with the hormone adsorbed to a matrix a situation more or less comparable to that in tissue sections. It is concluded that specificity testing of antisera applied in endocrine immunocytochemical studies can best be achieved by application of the ELISA method in combination with appropriate tissue controls.

The identification of peptide hormone secreting cells in tissue sections by immunocytochemistry is dependent on the specificity of the applied antibodies. This specificity is usually assessed by immunodiffusion or radioimmunoassay and confirmed by absorption of the antiserum with the target hormone antigen as a control in the immunostaining procedure. These procedures however are often insufficient to determine all wanted and unwanted immunoreactivities. Immunodiffusion techniques are insufficiently sensitive. The comparatively strong concentrations of antiserum required for immunocytochemistry may reveal cross-reactivities with related peptide hormones that are of little consequence in the dilutions applied for quantitative radioimmunoassay of plasma fluids or tissue extracts. Unless synthetic antigens are available for absorption of the antiserum, the specificity of this control procedure is illusory. For example, growth hormone antigen extracted from human post-mortem pituitaries used as an immunogen to raise growth hormone antibodies may be contaminated with a trace of prolactin. Absorption of the antiserum with this antigenic extract will fail to demonstrate the cross-reactivity to prolactin in immunostained sections.

Ideally, the cross-reactivities of peptide hormone antisera should be checked on serial concentrations of antigen in a solid phase mimicking the situation in tissue sections.¹ For this reason I have applied an

indirect method using an enzyme-linked immunosorbent assay (ELISA) in which the hormone antigens are adsorbed on to the walls of the cups of polystyrene microtitre plates.^{2,3} Serial dilutions of the antisera are added to the cups and after incubation positive reactivities are demonstrated by the addition of a peroxidase-labelled anti-IgG and the development of a peroxidase-dependent chromogenic reaction. The method proved to be sensitive and adequate and revealed unexpected cross-reactivities. The quantitative aspects indicated the degree of dilution of antiserum required to annul trace cross-reactivities and the necessity to discard or further refine antisera in which cross-reactivities were marked. In this paper the method is illustrated by the investigation of the immunoreactivities of antisera against the pituitary hormones prolactin and growth hormone and between portions of the pro-opiocortin molecule—namely, γ_3 -MSH, ACTH and β -LPH (Fig. 1). The results are compared with results of immunohistochemical staining.

Material and methods

MATERIAL

Hormones

Preparations of human prolactin, growth hormone and β -LPH were donated by Dr PJ Lowry. These hormones were obtained by extraction and purification of post-mortem pituitaries.^{4–6} Synthetic

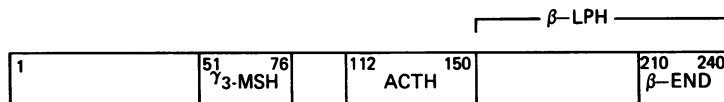


Fig. 1 Diagram of pro-opiomelanocortin

γ_3 -MSH was donated by Dr N Ling. Porcine 1-39 ACTH was purchased from Armour (Acthar) and synthetic 1-24 ACTH (Synacthen) was purchased from Ciba.

Antisera

These were all raised in rabbits. The antisera against human prolactin, growth hormone, synthetic β -endorphin and porcine ACTH were donated by Dr PJ Lowry. The γ_3 -MSH antiserum was donated by Prof LH Rees. These antibodies do not show non-specific immunoreactivities in radioimmunoassay. Since the complete amino acid sequence of β -endorphin is present in β -LPH (Fig. 1), the β -endorphin antiserum is reactive with β -LPH as well. Peroxidase-labelled porcine antirabbit IgG was purchased from Dako.

METHODS

Enzyme-linked immunosorbent assay (ELISA)

An indirect method was used^{2,3} as follows:

- (i) Disposable polystyrene microtitre plates (Dynatech) were washed in 0.1 mol/l HCl and distilled water and coated with the hormones mentioned above by adding to each cup 50 μ l of the hormone solution (1 μ g/ml) in 0.1 mol/l sodium carbonate buffer (pH 9.6) with 0.02% sodium azide, and incubating the plates at 37°C in a moist atmosphere for 3 h. Plates containing the hormone solution were stored at 4°C until use.
- (ii) Before assay the plates were washed three times for 5 min each with PBSA, composed of 0.01 mol/l phosphate buffer (pH 7.4), 0.15 mol/l NaCl and 1% bovine serum albumin (type V, Sigma).
- (iii) The plates were then incubated at 37°C for 30 min with 50 μ l per cup of serial dilutions of the antisera in PBSA.
- (iv) Washed three times for 5 min each with PBSA.
- (v) Incubated at 37°C for 30 min with 50 μ l per cup of porcine antirabbit IgG conjugated with peroxidase diluted to 1/200 in PBSA.
- (vi) Washed again three times for 5 min each with PBSA.
- (vii) Finally incubated with 100 μ l per cup of the enzyme substrate solution at room temperature. The reaction was stopped after about one hour by addition of 25 μ l 1 N NaOH to each cup.

The substrate used was a mixture of

5-aminosalicylic acid (5-AS, Merck) and H₂O₂ prepared according to Ruitenberg *et al*⁷ as follows: 80 mg of 5-AS were dissolved in 100 ml hot distilled water and filtered. Immediately before use the pH of this stock solution was brought to 6.0 with 1 N NaOH, and 25 μ l of 30% H₂O₂ was added. The amount of brown reaction product was evaluated visually. The optimal quantity of hormone to be coupled to the plates and the dilution of peroxidase-labelled IgG were determined by chess-board titration. Following this procedure optimal sensitivity was achieved when 10 to 100 ng of the hormones were coated per cup (Fig. 2).

Immunocytochemistry

Four μ m sections of formalin-fixed paraffin-wax embedded tissues of normal human pituitaries and pituitary adenomas from patients with either Cushing's disease, acromegaly or a prolactinoma were subjected to an indirect immunocytochemical method with peroxidase-labelled antibodies.^{3,8} After exposure to the antibodies the peroxidase-labelled hormone-antibody complexes were visualised with an enzyme substrate medium 3,3'-diaminobenzidine (Sigma) and H₂O₂ in Tris buffer.

Controls

These included direct addition of the peptide hormone antigens to the antisera and incubation overnight at 4°C.

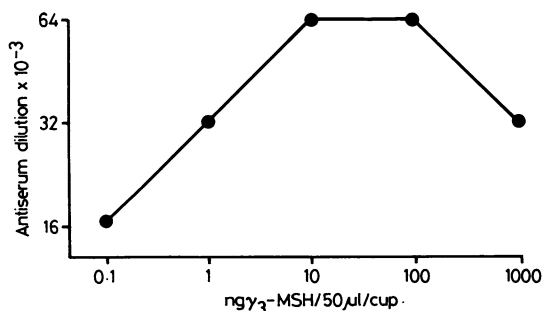


Fig. 2 Effect of the quantity of γ_3 -MSH coated to the microtitre plate on the sensitivity of the ELISA method. Optimal sensitivity is achieved when 10 to 100 ng of this peptide are coated per cup.

Immunoreactivities of antisera

	<i>Immunoreactivity determined with the ELISA technique</i>					<i>Cell type stained with immunocytochemistry</i>
	<i>GH</i>	<i>PRL</i>	<i>γ_3-MSH</i>	<i>ACTH</i>	<i>β-LPH</i>	
anti-GH	32 000*	4000	nil	200	nil	Somatotroph
anti-PRL	nil	16 000	nil	nil	200	Lactotroph
anti-GH pretreated with GH	nil	nil	nt	nt	nt	
anti- γ_3 -MSH	nil	nil	64 000	2000	8000	Corticotroph
anti-ACTH	nil	nil	nil	3200	<200	Corticotroph
anti- β -endorphin	nil	nil	nil	nil	8000	Corticotroph
anti- γ_3 -MSH pretreated with 1-24 ACTH	nil	nil	32 000	nil	nil	Corticotroph

*Reciprocal of antiserum dilution at which immunoreactivity is still present.

Results

The results of the investigations are summarised in the Table. With the ELISA technique all the antisera showed distinct immunoreactivities with the hormones against which they were raised. The growth hormone antiserum showed additional cross-reactivity with prolactin and to a moderate degree with ACTH. The cross-reactivity of this antiserum with prolactin was quenched by pretreatment of the antiserum with excess growth hormone (10 μ g/ml antiserum diluted to 1/1000 in PBSA). The prolactin antiserum showed some cross-reactivity with β -LPH. The γ_3 -MSH antiserum showed cross-reactivity with both ACTH and β -LPH. This cross-reactivity was quenched after pretreatment with synthetic 1-24 ACTH (10 μ g/ml antiserum diluted to 1/1000 in PBSA). The ACTH antiserum showed scanty cross-reactivity with β -LPH. The β -endorphin antiserum was reactive with β -LPH only.

With immunocytochemistry, all the antisera were reactive with only those cells in normal and adenomatous pituitary tissues of which it could be assumed by comparison with adjacent sections and on clinical data, that they contained the hormone against which the antiserum was raised (Table; Figs. 3 and 4). The final antiserum dilution appropriate for immunocytochemistry was in all instances about five times lower than the highest dilution at which immunoreactivity was still present with the ELISA method.

Discussion

The use of immunocytochemistry in endocrine investigations requires a twofold specificity of the relevant antiserum: the antiserum should only react with the hormone against which the antiserum is raised, and the binding should be restricted to unique antigenic determinants that are not present on other portions of the prohormone from which the



Fig. 3 Human pituitary containing a prolactinoma, immunostained with the growth hormone antiserum. Distinct growth hormone immunoreactivity is present in pre-existent pituitary cells, but not in adenoma cells. Diaminobenzidine $\times 400$

relevant hormone is derived. In our experience the indirect method of the enzyme-linked immunosorbent assay (ELISA) is more suitable than immunodiffusion, radioimmunoassay or incubation of tissue sections with absorbed antisera to assess these specificities. The method is cheap, simple and rapid to perform. It does not use radionuclides. It does not require large quantities of usually expensive hormones. It is also sensitive to antibodies of low titre and avidity. It is quantitative for the anti-

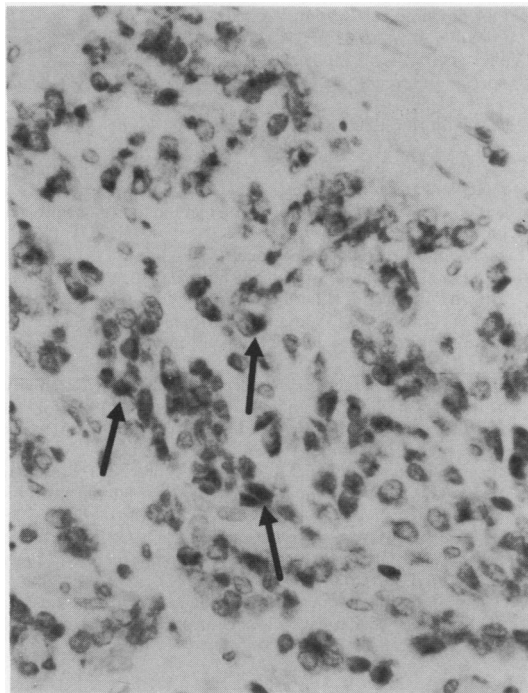


Fig. 4 Higher magnification of the prolactinoma shown in Fig. 3, immunostained with the prolactin antiserum. Distinct prolactin immunoreactivity is present in adenoma cells. Diaminobenzidine $\times 1000$

body. Finally, it represents, with the hormone attached to a matrix, a situation more or less comparable to that of the hormone fixed in a tissue section.

For visual reading of ELISA results we prefer 5-aminosalicylic acid as chromogenic substrate in the enzyme reaction. This compound is partially insoluble and therefore not very suitable for photometric reading. Since another peroxidase substrate, *o*-phenylene diamine, is completely soluble and renders a high extinction coefficient, this compound is recommended as the preferable substrate for photometric reading.² However, the dark-brown reaction product obtained after 5-aminosalicylic acid enables easier differentiation by eye between specific and background coloration than the orange reaction product obtained after *o*-phenylene diamine.

In our hands, the optimal amount of hormone to be coated to the microtitre plates usually varies between 10 to 100 ng/cup. As illustrated in Fig. 2, coating of more than the optimal quantity results in a decrease of sensitivity, which is possibly due to occurrence of physical hindrance to the entry of the antibodies.

Because of its high sensitivity, the ELISA method may reveal scanty cross-reactivities that are not relevant for immunocytochemical staining. For instance, the growth hormone and prolactin antisera showed some cross-reactivity with respectively ACTH and β -LPH, but were not reactive with corticotrophic cells in tissue sections. On the other hand, cross-reactivities demonstrated with the ELISA method may not be due to the presence of non-specific antibodies in the antiserum, but instead be caused by contamination of the hormone preparation used for the coating and testing with the hormone with which the antiserum is cross-reactive. This is most likely the explanation for the finding that with the ELISA method the growth hormone antiserum showed distinct cross-reactivity with prolactin, whereas no reaction of this antiserum with prolactin cells in tissue sections was observed (Figs. 3 and 4).

Pretreatment of the growth hormone antiserum with excess growth hormone quenched the cross-reactivity with prolactin in the ELISA method, indicating that the cross-reaction of this antiserum with the prolactin antigen was not due to the presence of prolactin antibodies in the growth hormone antiserum, but caused by contamination of the prolactin antigen used for the coating and testing in the ELISA method with growth hormone. This confirms that antigens obtained by extraction are often contaminated and therefore not very useful for absorption controls on tissue sections.

Discrepancies between results obtained with ELISA and immunocytochemistry as is demonstrated for growth hormone and prolactin, are less clear when the peptides with which the antiserum is cross-reactive are localised in the same cell as the specific peptide. For instance, staining of corticotrophic cells with antisera against portions of the pro-opiocortin molecule (Fig. 1) may be due either to presence of the relevant portion of pro-opiocortin or to cross-reaction with other portions which share antigenic determinants. This is obviously the case with the γ_3 -MSH antiserum, which was reactive with ACTH and β -LPH as well with ELISA. γ_3 -MSH, ACTH and β -LPH have a four amino acid sequence in common, which in ACTH is present at the 6-9 position. Pretreatment of the γ_3 -MSH antiserum with synthetic 1-24 ACTH quenched the cross-reactivity with ACTH and β -LPH, but did not affect the reaction of the antiserum with corticotrophic cells in tissue sections. This illustrates the suitability of the ELISA technique to demonstrate that the pretreatment with 1-24 ACTH was sufficient to neutralise the cross-reactivity with ACTH and β -LPH and that the final immunoreaction of the γ_3 -MSH antiserum with corticotrophic cells is due to the presence

of γ_3 -MSH immunoreactivity in these cells.

As is the case for all methods applied in specificity testing of antibodies, a limitation of the ELISA method is that only immunoreactivities with peptides that are known and available for testing can be studied. Even when monoclonal antibodies are used, reactivities with unknown peptides usually remain hidden, unless the results of the ELISA and immunocytochemical staining are correlated with clinical data and biological and chromatographic analysis of tissue extracts with which the antibodies are reactive. As even this approach may be insufficient to demonstrate non-specificities, it is highly questionable whether specificity can be proven in immunocytochemistry.⁹ I feel however that within the limits of imperfection, assessment of reactivities of antisera to be used in endocrine immunocytochemistry can ultimately best be achieved when the ELISA method is used in combination with appropriate tissue controls.¹⁰

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Requests for reprints to: Dr AC Nieuwenhuijzen Kruseman, Department of Endocrinology, University Hospital, Rijnsburgerweg 10, 2333 AA Leiden, The Netherlands.